

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Patent Application of:	Group Art Unit: 1631
Applicant: Michael W. PANTOLIANO <i>et al.</i>	Examiner: Shubo (Joe) Zhou
Serial No.: 10/821,274	
Filed: April 7, 2004	
Title: MICROPLATE THERMAL SHIFT ASSAY APPARATUS FOR LIGAND DEVELOPMENT AND MULTI-VARIABLE PROTEIN CHEMISTRY OPTIMIZATION	<p>Electronically filed on April 3, 2006.  Vicki Andrews</p>

**RESPONSE**

Mail Stop Amendment  
Commissioner for Patents  
PO Box 1450  
Alexandria, Virginia 22313-1450

Sir:

This is a response to the Office Communication mailed March 2, 2006. The one-month deadline for filing a response falls on April 2, 2006 (a Sunday). Therefore, Applicants believe that this response is being timely filed. In the event that there are any fees required in connection with this response, please charge any necessary fee to Deposit Account No. 23-2415, referencing Docket No. 30923-702.306.

**Amendments to the Specification** begin on page 2 of this paper.

**Remarks** begin on page 9 of this paper.

**Amendments to the Specification:**

Please replace the Title of the Application as follows:

~~"MICROPLATE THERMAL SHIFT ASSAY APPARATUS FOR LIGAND DEVELOPMENT AND MULTI-VARIABLE PROTEIN CHEMISTRY OPTIMIZATION"~~

**"METHOD FOR LIGAND DEVELOPMENT"**

Please replace paragraph [0007] (page 2, line 3) as follows:

[0007] The rate of combinatorial library synthesis is accelerated by automating compound synthesis and evaluation. For example, DirectedDiversity® DIRECTEDDIVERSITY® is a computer based, iterative process for generating chemical entities with defined physical, chemical and/or bioactive properties. The DirectedDiversity® DIRECTEDDIVERSITY® system is disclosed in U.S. Pat. No. 5,463,564, which is herein incorporated by reference in its entirety.

Please replace paragraph [0047] (page 16, lines 12-24) as follows:

[0047] FIG. 3 shows the results of a microplate thermal shift assay for ligand binding to the active site of human  $\alpha$ -thrombin (with fluorescence emission as the experimental signal). The lines drawn through the data points represent non-linear least squares curve fits of the data using the equation shown at the bottom of the figure. There are five fitting parameters for this equation of  $y(T)$  vs.  $T$ : (1)  $y_n$ , the pre-transitional fluorescence for the native protein; (2)  $y_u$ , the post-transitional fluorescence for the unfolded protein; (3)  $T_m$ , the temperature at the midpoint for the unfolding transition; (4)  $\Delta H_u$ , the van't Hoff unfolding enthalpy change; and (5)  $\Delta C_{pu}$ , the change in heat capacity upon protein unfolding. The non-linear least squares curve fitting was accomplished using KaleidaGraph™ KALEIDAGRAPH™ 3.0 software (Synergy Software, Reading Pa.), which allows the five fitting parameters to float while utilizing Marquardt methods for the minimization of the sum of the squared residuals.

Please replace paragraph [0110] (page 26, line 30 to page 27, line 20) as follows:

[0110] The term "fluorescence probe molecule" refers to a fluorophore, which is a fluorescent molecule or a compound which is capable of binding to an unfolded or denatured receptor and, after excitement by light of a defined wavelength, emits fluorescent energy. The term fluorescence probe molecule encompasses all fluorophores. More specifically, for proteins, the term encompasses fluorophores such as thioinosine, and N-ethenoadenosine, formycin, dansyl derivatives, fluorescein derivatives, (6-propionyl-2-dimethylamino)-naphthalene (PRODAN), 2-anilinonaphthalene, and N-arylamino-naphthalene sulfonate derivatives such as 1-anilinonaphthalene-8-sulfonate (1,8-ANS), 2-anilinonaphthalene-6-sulfonate (2,6-ANS), 2-aminoanaphthalene-6-sulfonate, N,N-dimethyl-2-aminonaphthalene-6-sulfonate, N-phenyl-2-aminonaphthalene, N-cyclohexyl-2-aminonaphthalene-6-sulfonate, N-phenyl-2-aminonaphthalene-6-sulfonate, N-phenyl-N-methyl-2-aminonaphthalene-6-sulfonate, N-(o-tolyl)-2-aminonaphthalene-6-sulfonate, N-(m-tolyl)-2-aminonaphthalene-6-sulfonate, N-(p-tolyl)-2-aminonaphthalene-6-sulfonate, 2-(p-toluidinyl)-naphthalene-6-sulfonic acid (2,6-TNS), 4-(dicyanovinyl) julolidine (DCVJ), 6-dodecanoyl-2-dimethylaminonaphthalene (LAURDAN), 6-hexadecanoyl-2-((2-(trimethylammonium)ethyl)methyl)amino)naphthalenechloride(PATMAN), nile red, N-phenyl-1-naphthylamine, 1,1-dicyano-2-[6-(dimethylamino)naphthalen-2-yl]propene (DDNP), 4,4'-dianilino-1,1-binaphthyl-5,5-disulfonic acid (bis-ANS), and ~~D~~apoxy<sup>TM</sup> DAPOXYL<sup>TM</sup> derivatives (Molecular Probes, Eugene, Oreg.). Preferably for proteins, the term refers to 1,8-ANS or 2,6-TNS.

Please replace paragraph [0162] (page 36, line 16 to page 37, line 2) as follows:

[0162] A fluorescence emission imaging system can be used to monitor the thermal denaturation of a target molecule or a receptor. Fluorescence emission imaging systems are well known to those skilled in the art. For example, the AlphaImager<sup>TM</sup> ALPHAIMAGER<sup>TM</sup> Gel Documentation and Analysis System (Alpha Innotech, San Leandro, Calif.) employs a high performance charge coupled device camera with 768 x 494 pixel resolution. The charge coupled device camera is interfaced with a computer and images are analyzed with Image analysis software<sup>TM</sup>. The ChemilImager<sup>TM</sup> CHEMIIMAGER<sup>TM</sup> (Alpha Innotech) is a cooled charge

coupled device that performs all of the functions of the AlphaImager™ ALPHAIMAGER™ and in addition captures images of chemiluminescent samples and other low intensity samples. The ChemiImager™ CHEMIIMAGER™ charge coupled device includes a Pentium processor (1.2 Gb hard drive, 16 Mb RAM), AlphaEase™ ALPHAEASE™ analysis software, a light tight cabinet, and a UV and white light trans-illuminator. For example, the MRC-1024 UV/Visible Laser Confocal Imaging System (BioRad, Richmond, Calif.) facilitates the simultaneous imaging of more than one fluorophore across a wide range of illumination wavelengths (350 to 700 nm). The Gel Doc 1000 Fluorescent Gel Documentation System (BioRad, Richmond, Calif.) can clearly display sample areas as large as 20 x 20 cm, or as small as 5 x 4 cm. At least two 96 well microplates can fit into a 20 x 20 cm area. The Gel Doc 1000 system also facilitates the performance of time-based experiments.

Please replace paragraph [0223] (page 55, lines 6-14) as follows:

[0223] Some fluorescent plate readers employ lasers for excitation in the visible region of the electromagnetic spectrum. For example, the FluorImager™ FLUORIMAGER™ (Molecular Dynamics, Palo Alto, Calif.) is such a device. This technology is useful when using fluorescent dyes that absorb energy at around 480 nm and emit energy at around 590 nm. Such a dye could then be excited with the 488 nm illumination of standard argon, argon/krypton lasers. For example, 1,1-dicyano-2-[6-(dimethylamino)naphthalen-2-yl]propene (DDNP) is such a dye. The advantage in using a laser is that a laser is characterized by very high intensity light, which results in an improved signal to noise ratio.

Please replace paragraph [0237] (page 59, line 25 to page 60, line 3) as follows:

[0237] Spectral emission 2918 from samples 2910 is transmitted from photomultiplier tube 2904. Electrical output 3140 connects photomultiplier tube 2904 to electric connection 2902. Electric connection 2902 connects electrical output 3140 to computer 2914. Driven by suitable software, computer 2914 processes the spectral emission signal from samples 2910. Exemplary software is a graphical interface that automatically analyzes fluorescence data obtained from samples 2910. Such software is well known to those of ordinary skill in the art. For example, the CytoFluor™ II

CYTOFLUORTMII fluorescence multi-well plate reader (PerSeptive Biosystems, Framingham, Mass.) utilizes the Cytoeale™ CYTOCALCTM Data Analysis System (PerSeptive Biosystems, Framingham, Mass.). Other suitable software includes, MicreSoft MICROSOFTTM Excel or any comparable software.

Please replace paragraph [0271] (page 70, lines 4-15) as follows:

[0271] Using the computer controlled process DirectedDiversity® DIRECTEDDIVERSITY® (see U.S. Pat. No. 5,463,564), scientists at 3-Dimensional Pharmaceuticals, Inc. have generated a combinatorial library of compounds directed at the active site of human  $\alpha$ -thrombin.

Approximately 400 compounds were synthesized and assayed by a conventional spectrophotometric kinetic assay in which succinyl-Ala-Ala-Pro-Arg-p-nitroanilide (Bachem, King of Prussia, Pa.) served as substrate. Five of these compounds, which are characterized by K<sub>i</sub>'s that span almost four orders of magnitude in binding affinity, were used to test the range and limits of detection of the thermal shift assay. These five proprietary compounds are listed in Table 3, along with the K<sub>i</sub> for each respective compound, as measured by the kinetic assay (last column). K<sub>i</sub>'s for these compounds ranged from 7.7 nM for 3dp-4026 to 20.0  $\mu$ M for 3dp-3811.

Please replace paragraph [0272] (page 70, line 16 to page 71, line 15) as follows:

[0272] A stock human  $\alpha$ -thrombin solution (1.56 mg/mL) from Enzyme Research Labs was first diluted to 0.5 mg/mL (11  $\mu$ M) with 50 mM Hepes, pH 7.5, 0.1 M NaCl (assay buffer, unless mentioned otherwise), and stored on ice. The five ligands (recrystallized solids characterized by mass spectrometry and NMR) were accurately weighed out to be 1.5 to 2.0 mg and dissolved in 1.0 mL of 100% DMSO so that the concentration was between 1.8 and 3.8 mM. A 96 well V-bottom Costar microplate was then set up such that 100  $\mu$ L of the 11  $\mu$ M human  $\alpha$ -thrombin solution was pipetted into wells A1 through A6. This was followed by the addition of 2  $\mu$ L of 3dp-3811 into well A2, 2  $\mu$ L of 3dp-3959 into well A3, 2  $\mu$ L of 3dp-4077 into well A4, 2  $\mu$ L of 3dp-4076 into well A5, 2  $\mu$ L of 3 dp-4026 into well A6, and 2  $\mu$ L of 100% DMSO into control well A1. The contents were mixed by repeated uptake and discharge using a 100  $\mu$ L pipette tip. Finally, one drop of mineral oil (Sigma, St. Louis, Mo.) was added on top of the wells to reduce

evaporation of samples at elevated temperatures. The microplate was then placed on heating block 4 of a Reboeycler™ ROBOCYCLER™ temperature cycler (Stratagene, La Jolla, Calif.), set at 25°C, for 1 minute. The plate was then placed into a SPECTRAmax™ SPECTRAMAX™ 250 spectrophotometer (set to 30°C) and the absorbance at 350 nm was measured for each sample. This reading served as the blank or reference from which all the other readings at higher temperatures were compared. The assay was initiated by setting heating block 1 to 38°C, programming the temperature cycler to move the microplate to heating block 1, and keeping the microplate there for 3 minutes. Following the equilibration at 38°C, the plate was moved to the 25°C block (Block 4) for 30 seconds, inserted in the spectrophotometer, and absorbance was read at 350 nm. The microplate was then put back into the temperature cycler and was moved to heating block 2, which had been pre-equilibrated at 40° C. After 3 minutes at 40°C, the plate was returned to 25°C (on block 4) for 30 seconds, and was returned to the spectrophotometer for a measurement of absorbance at 350 nm. This process was repeated 18 more times until the temperature had been raised to 76° C in 2°C increments. After subtraction of the blank absorbance ( $A_{350}$  at 25°C), turbidity, reflected in the absorbance value, was plotted as a function of temperature. The thermal denaturation curves for this experiment are shown in Figure 1.

Please replace paragraph [0290] (page 81, line 20 to page 82, line 7) as follows:

[0290] In order to further demonstrate the cross target utility of the microplate thermal shift assay, another enzyme, Factor D, was tested for its ability to undergo thermal unfolding transitions. Factor D is an essential serine protease involved in the activation of the alternative pathway of the complement system, the major effector system of the host defense against invading pathogens. Factor D was purified from the urine of a patient with Fanconi's syndrome (Narayana et al., J. Mol. Biol. 235:695-708 (1994)) and diluted to 4  $\mu$ M in assay buffer (50 mM Hepes, pH 7.5, 0.1 M NaCl). The assay volume was 10  $\mu$ L and the concentration of 1,8-ANS was 100  $\mu$ M. The experiment was carried out using 15  $\mu$ L round bottom dimple plates (an 8 x 12 well array). The protein was heated in two 30 degree increments between 42° C to 62° C, using a Reboeycler™ ROBOCYCLER™ temperature cycler. After each heating step, and prior to fluorescence scanning using the CytoFluor™ II CYTOFLUORTM II fluorescence plate reader the sample was cooled to 25° C (see Example 1). The non-linear least squares curve fitting and other

data analysis were performed as described for FIG. 3. The results of the microplate thermal shift assay of Factor D is shown in FIG. 5 and reveal a thermal unfolding transition that occurs near 324 K (51° C) for the unliganded form of the protein. No reversible ligands of significant affinity are known for Factor D. The results in FIG. 5 show that the microplate thermal shift assay can be used to screen a library of compounds for Factor D ligands. The results in FIG. 5 also show that the microplate thermal shift assay is generally applicable to any target molecule.

Please replace paragraph [0291] (page 82, lines 10-22) as follows:

[0291] Human Factor Xa, a key enzyme in the blood clotting coagulation pathway, was chosen as yet another test of the cross target utility of the microplate thermal shift assay. Factor Xa was purchased from Enzyme research Labs (South Bend, Ind.) and diluted to 1.4  $\mu$ M in assay buffer (50 mM Hepes, pH 7.5, 0.1 M NaCl). The assay volume was 100  $\mu$ L and the concentration of 1,8-ANS was 100  $\mu$ M. The protein was heated in two degree increments between 50° C to 80° C using a Rebeyeler™ ROBOCYCLER™ temperature cycler. After each heating step, prior to fluorescence scanning using the CytoFluor™ CYTOFLUORTMII fluorescence plate reader, the sample was cooled to 25° C (see Example 1). The results of a microplate thermal shift assay of Factor Xa is shown in FIG. 6. A thermal unfolding transition was observed at 338K (65° C). Data analysis was described as described for FIG. 3. The results in FIG. 6 show that the microplate thermal shift assay of protein stability is generally applicable to any target molecule.

Please replace paragraph [0300] (page 83, line 22 to page 84, line 10) as follows:

[0300] The measurements shown in FIG. 7 were obtained using the CytoFluor™ CYTOFLUORTMII fluorescence plate reader (PerSeptive Biosystems, Framingham, Mass.). In the experiment, the excitation wavelength of light was 360 nm and the emission was measured at 460 nm. The microplates employed for this miniaturized assay were either the conventional polycarbonate V-bottom 96 well plate (Stratagene, or Costar) or polycarbonate plates that contain 15  $\mu$ L dimples in an 8 x 12 array (Costar plate lids). In the reaction, the concentration of human  $\alpha$ -thrombin was  $\mu$ M in assay buffer (50 mM Hepes, pH 7.5, 0.1 M NaCl). The assay volume was 5  $\mu$ L and the concentration of 1,8-ANS was 100  $\mu$ M. The protein was heated in two degree

increments between 44° C to 64° C using a Reboeyeler™ ROBOCYCLER™ temperature cycler. After each heating step, and prior to fluorescence scanning using the CytoFluor™II CYTOFLUORTMII fluorescence plate reader the sample was cooled to 25° C for 30 seconds (see Example 1). The non-linear least squares curve fitting and other data analysis were performed as described for FIG. 3.

Please delete paragraphs [0334]; [0335] and [0336].

Please replace paragraph [0339] (page 97, line 16 to page 98, line 10) as follows:

[0339] A 2  $\mu$ M solution of human  $\alpha$ -thrombin was prepared in 50 mM Hepes, pH 7.5, 0.1 M NaCl by diluting a 34  $\mu$ M stock solution (1:17) of purified human  $\alpha$ -thrombin (Enzyme Research Labs, Madison, Wis.). The human  $\alpha$ -thrombin solution also contained 100  $\mu$ M 1,8-ANS. 100  $\mu$ L of the human  $\alpha$ -thrombin-1,8-ANS solution was aliquoted into each of twelve wells of a single row (row A) of a V-bottom polycarbonate microplate (Costar). A gradient block (Reboeyeler™ ROBOCYCLER™, Stratagene) was used to heat the twelve samples, from 44 to 66° C, across the rows of the microplate. i.e. a temperature gradient of 2° C per well was established. Thus, well A1 was at 66° C and well A12 was at 44° C. The control solution that contained 100  $\mu$ M 1,8 ANS in the same buffer (no protein) was placed in each of wells B1 to B12. After adding a drop of mineral oil to each well to prevent evaporation, the plate was heated on the gradient block for 3 min. The contents of each well were then allowed to reach room temperature and transferred to a flat bottom microplate. In this experiment, no filters were employed to narrow the excitatory wavelength to 7360 nm and the emission wavelength to about 460 nm, which are optimal wavelengths for the 1,8 ANS fluorophore. The flat bottom plate was then placed on the near UV transillumination box and the CCD camera was used to measure the amount of emitted light. The plate was also read using a conventional fluorescence plate reader (CytoFluor™II CYTOFLUORTMII), in order to compare the results obtained by the two different detection methods. The results for the two detection methods are plotted in FIG. 40. The results in FIG. 40 show that the CCD camera is useful as a fluorescence emission detector for monitoring the unfolding of a protein in the microplate thermal shift assay.

**REMARKS**

Pursuant to 37 C.F.R. 1.121, only the corrected section of the non-compliant amendment has been re-submitted in its entirety.

The shortened statutory period for reply expires on April 2, 2006. Therefore, no fee is believed to be due in connection with this submission. However, if the Office determines that any fee is due, please charge Deposit Account No. 23-2415, referencing docket no. 30923-702.306.

If the Office believes, for any reason, that personal communication will expedite prosecution of this application, the Office is invited to telephone the undersigned at (858) 350-2337.

Respectfully submitted,

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